Construction and analysis of a subtracted cDNA library of *Betula platyphylla* female inflorescence

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Abstract: Female inflorescence of *Betula platyphylla* was sampled at an interval of each two days to analyze the background of gene expression in floral phase. On the basis of SMART strategy, the driver cDNA was obtained from total RNA of the last sample and the tester cDNA was from that of the others by RT-PCR which were subsequently used to construct a subtracted cDNA library. The result of the ESTs (expression sequence tags) blastX showed that the genes in the subtracted cDNA library could be mainly clustered into 5 groups related to metabolism, transportation and signal transduction, cell cycle, stress response, and regulation. The relationship between gene expression and development was also discussed.

Keywords: *Betula platyphylla*; Subtracted cDNA library; SMART
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Introduction

Genetic and molecular studies have uncovered a large number of genes that control different steps in flower development including flowering time, flower meristem, and organ identity. In particular, the ABC model has been proposed for the specification of floral organ identity (Coen and Meyerowitz 1991), and the discovery of D and E function genes perfected the regulatory network about floral development (Colombo *et al.* 1995; Pelaz *et al.* 2000). At present a great advance has been made to elucidate the genetic mechanism of floral development in many species.

Birch is a kind of eminent tree species in architecture, furniture, and paper making. At present a breakthrough has been made in accelerating genetic breeding by the way of manpower early flowering in which process the physiological information was monitored (Su *et al.* 2000). However, few of researches about the gene expression pattern of flowering were reported, and until recently a few genes were found for regulating floral development in *Betula pendula* which have the function as *AP*1 (Elo *et al.* 2001), *PI* (Jarvinen *et al.* 2003), *SEP*, *AG* (Lemmetyinen *et al.* 2004) in *Arabidopsis* or *SBP* (Lannenpaa *et al.* 2004) in Snapdragon. In this study, we report some new study in floral development process of *Betula platyphylla*, which will found a basis for future work of cloning genes by the RACE (rapid amplification of cDNA ends) method.

Material and method

Female inflorescence was sampled from birch forest in yard of Northeast Forestry University, at an interval of each two days from April 30 to June 2, 2004. The samples were quickly frozen with liquid nitrogen, and kept at -70℃ before extracting the total RNA.

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Total RNA isolation

The sample from the last time was used as origin of driver cDNA of *Betula platyphylla*, while the mixture from the other time was for the tester cDNA.

The total RNA of *Betula platyphylla* was isolated by SDS/phenol method (Frederick *et al.* 1995).The integrity of total RNA was detected by the electrophoresis on a 0.8% agrose/EB gel, and its quality and amount were detected with UV-spectrometer (Eppendorf).

cDNA synthesis

First-strand cDNA synthesis was finished by the SMART™ RACE cDNA Amplification Kit.

cDNA amplification

PCR system includes 2.5 μL 10×LA buffer, 4 μL dNTP (2.5 mM each), 0.5 μL primer (20 μM), 1 μL First-strand cDNA, 1.5 U LA-Taq polymerase (TAKARA), and sterile H_2O to total 25 μL.

PCR was performed at 94℃ for 1 min, then 94℃ for 30 s, 68℃ for 30 s, 72℃ for 5 min for 18 circles, and with post incubation at 72℃ for 5 min. The PCR product was purified with gel purification kit (Promega), and the 3 μL purified PCR product was detected by the electrophoresis of 1.0% agrose/EB gel.

cDNA digestion by Rsa I, adaptor ligation to tester cDNA, and hybridization

The procedure of Clontech PCR-Select™ cDNA Subtraction Kit was referenced.

Amplification

According to the procedure of Clontech PCR-Select™ cDNA Subtraction Kit, the diluted hybridization product was amplified for 25 circles with primer 1 by primary PCR, and diluted primary PCR product was amplified for 15 circles with nest primers in the second amplification. For T-A cloning, 15-min post incubation was adopted at 72℃.

After electrophoresis, small fragments shorter than 250 bp were discarded by gel cutting and the PCR product was purified with gel purification kit (Promega).

A subtracted cDNA library was constructed by the pGEM-T Vector System (Promega). After examination of ligation efficiency with M13 reverse primer and M13 forward primer, a total of 150 randomly selected clones were sequenced to check for redundancy, which was 14%.

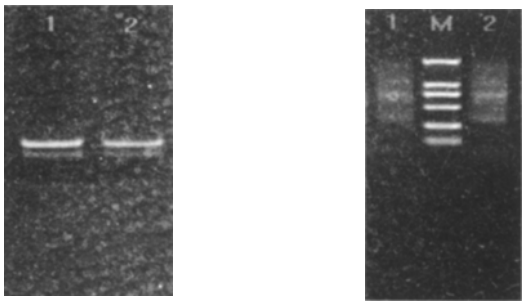
Result

Isolation of total RNA

The integrity of RNA was examined by electrophoresing samples on an agarose/EtBr gel. Total RNA typically exhibits two bright bands which correspond to ribosomal 28S and 18S RNA, respectively, with a ratio of intensities of about 1.5–2.5:1 (Fig.1). As starting material, the quality of the total RNA can meet the needs for constructing a subtracted cDNA library by SMART strategy.

Amplification of cDNA

cDNA amplified with 18 circles was detected on an agarose/EtBr gel, and both driver cDNA and tester cDNA appeared smearing with a few bright bands (Fig. 2) which may be the transcripts of housekeeping genes.



Examination of amplification of hybridization product

Examined on an agrose/EB gel, PCR product of fragments in the library appeared smearing with a size about 150–750 bp (Fig. 3) in which the fragments were easy to be cloned to T-vector.

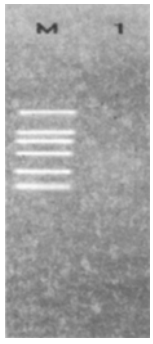


Fig. 3 Amplification of hybridization product

Lane M: DL-2000 marker; Lane 1: PCR product

Examination of ligation efficiency

The average length of inserting fragments can be calculated according to the result from Fig. 4, which is about 350 bp, longer than the average length of Rsa I digestion (250 bp) because of gel excising.

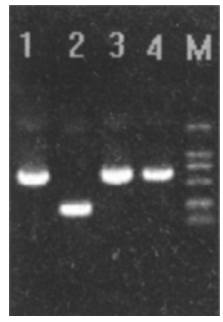


Fig. 4 Amplification of plasmids from random clones

Lane M: DL-2000 marker

Lane 1, 3, 4: Products of recombinants

Lane 2: 238 bp product of self-ligation,

Analysis of the ESTs

Among 150 clones, the ESTs (Expression sequence tags) with significant homology (E-value<$1e^{-10}$) and sequences in the public databases were mainly classified into 5 clusters listed as follows.

Cluster A:

Suggested function	EST number
Ribosomal protein	1
PSI I protein	2
Ribulose-1,5-bisphosphate carboxylase large subunit	3
ATPase proteolipid subunit	4
ATP citrate lyase	5
Isocitrate dehydrogenase	6
Mitochondrial malate dehydrogenase	7
Ubiquinone oxidoreductase	8
Alcohol dehydrogenase	9
Myo-inositol 1-phosphate synthase	10
Phosphoglycerate mutase	11
Acetolactate synthase	12
Acetyl-CoA carboxylase	13
Gdsl-motif lipase	14
Family II lipase EXL3	15
Lipoxygenase	16
Sphingolipid delta-8 desaturase	17
RNA polymerase beta I subunit	18
Glycyl tRNA synthetase	19
ATP-dependent Clp protease	20
Amine oxidase 1	21
SSR alpha subunit	22
Ubiquitin carrier protein E2	23
26s proteasome regulatory particle non- ATPase subunit8	24
Urease accessory protein G	25

The ESTs in cluster A reflect a background of gene expression in the process of floral development, which involves the process of photosynthesis, respiration, carbohydrates metabolism, lipid metabolism, and protein metabolism. Ubiquitin-dependent degradation of regulatory proteins can control many cellular processes, including cell cycle progression, morphogenesis, and signal transduction. Post-translational modification by ubiquitin targets many proteins for rapid degradation by the 26S proteasome. The E2 ubiquitin-conjugating enzyme is a component required for covalent attachment of ubiquitin onto lysine residues of the substrate (Galan & Peter 1999). Urease is a nickel-dependent metalloenzyme, and catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. Urease plays an im-

portant role in germination and nitrogen metabolism of seedling. It may function coordinately with arginase in the utilization of seed protein reserves during germination (Sirko & Brodzik 2000). Urease accessory protein G is involved into urease maturation i.e. the placing of essential nickel in the urease active site.

Cluster B:

Suggested function	EST number
ABC transporter	26
Nramp2	27
Protein transport factor	28
Amino acid transport protein	29
Lipid transfer protein precursor	30
Seven transmembrane protein Mlo4	31
Membrane protein	32
L-ascorbate oxidase	33
Protein phosphatase	34
Protein kinase	35
BRI1-KD interacting protein	36
NPG1 (No Pollen Germination 1)	37
EICBP4 (ethylene-induced calmodulin-binding protein 4)	38

Many ESTs in cluster B are related to the transmembrane transportation including ion, amino acid, protein and lipid. Three ATP binding cassette (ABC) transporter-like activities directed toward large amphipathic organic anions have recently been identified on the vacuolar membrane of plant cells. These are the Mg-ATP-energized, vanadate-inhibitable vacu olar accumulation of glutathione *S*-conjugates (GS conjugates), chlorophyll catabolites, and bile acids, respectively. *AtMRP2*, from *Arabidopsis thaliana* that encodes a multi-specific ABC transporter competent in the transport of both GS conjugates and chlorophyll catabolites (Lu *et al.* 1998; Liu *et al.* 2001). While protein kinase, BRI1-KD interacting protein, NPG1 and EICBP4 maybe is involved into signal transduction, especially NPG1 is a CaM-binding protein that mediates pollen germination and pollen tube growth. It is expressed with a pollen-specific pattern in *Arabidopsis thaliana*, and is likely to be a key protein in the calcium signaling pathway in mediating calcium changes during pollen germination and tip growth (Golovkin & Reddy 2003).

Cluster C:

Suggested function	EST number
Cdc20 protein	39
Microtubule associated protein	40
Exostosin family protein-like	41
Histone H_2A protein	42
Actin-related protein4	43
Knolle	44
SCC3	45
Endo-1,3(4)-beta-glucanase	46
Beta-glucosidase.	47

The ESTs in cluster C are mainly related to cell cycle. Histones are a major component of eukaryotic chromatin in association with DNA and nonhistone chromosomal proteins. These proteins and their mRNAs are usually synthesized in a cell cycle-dependent fashion at the beginning of *S* phase. This mode of synthesis is necessary to allow nucleosome formation on the duplicated DNA. Some histones are expressed in a non-cell cycle-dependent manner. In this case, mRNA of histone H_2A is differentially expressed during development and that it exhibits a distinct cell-specific pattern of expression (Koning *et al.* 1991; Xu 1999). Actin is a ubiquitous component of the plant cytoskeleton and participates in a number of important subcellular processes such as cell division plane localization, cell elongation, and cell shape determination. Actin is encoded by a gene family, and *ACT4* is expressed in mature pollen and young vascular tissue in *Arabidopsis* (McKinney *et al.* 2002). SCC3 is a subunit of cohesions, protein complexes associated with sister chromatids in mitotic cell, and its expression should have something with regulation of chromosomal behavior (Pelttari *et al.* 2001). KNOLLE protein is related to a family of membrane anchored proteins, the syntaxins, which are implicated in directing intracellular vesicle trafficking. KNOLLE protein is specifically expressed in mitotically dividing cells and in the cellularizing endosperm, mediating vesicle fusion in the plane of cell division, and was detected in mitotically dividing cells of various parts of the developing plant, including seedling root, inflorescence meristem, floral meristems and ovules, and the cellularizing endosperm (Lauber *et al.* 1997). Endo-1, 3 (4)-beta-D-glucosidase, beta-glucosidase is involved into the process of cell wall formation and decomposition, is highly expressed during fruit ripening.

Cluster D:

Suggested function	EST number
Manganese superoxide dismutase	48
Metallothionein	49
Cytochrome P450	50
Polyphenol oxidase PPO1	51

The ESTs in cluster D are mainly related to stress response. Expression of manganese superoxide dismutase appeared to be cross linked with the dehydration process during seed maturation. Metallothionein and cytochrome P450 can attach metal-ion, and play a role in decomposing toxin. PPO is an enzyme catalyzing the oxidation of *o*-diphenolic compounds to *o*-quinones as well as the hydroxylation of monophenols to *o*-diphenols. A role of foliar PPO in the defense against leaf-eating insects has been proposed and documented (Felton *et al.* 1989). Fruit commonly contains large amounts of PPO. However, the physiological function of PPO in fruit and other organs in healthy plants is still uncertain.

Cluster E:

Suggested function	EST number
DOF2 (DNA binding with one finger2) protein	52
Nucleoid DNA-binding-like protein	53
TFIID component TAF2	54
Elongation factor 1-alpha 1	55
Eukaryotic initiation factor 4B	56
GHMYB25	57
SBP (*squamosa* promoter binding protein)	58
HEN4 (Hua enhancer 4)	59

The ESTs in cluster E belong to regulatory factors including universal regulators and specific regulators. Dof domain proteins function as a transcriptional activator or a repressor were involved in diverse plant-specific biological processes and played critical roles as transcriptional regulators in plant growth and development (Yanagisawa 2002). TFIID component TAF2, elongation factor 1-alpha 1, eukaryotic initiation factor 4B and nucleoid DNA-binding-like protein are universe regulators, only nucleoid DNA-binding-like protein is localized in chloroplast. GHMYB25 is an MYB type transcription factor in *Gossypium hirsutum* that controls pigment formation, and there is a gene down-stream encoding isoflavone reductase in this subtracted cDNA library. SBP is a transcription factor regulating expression of *API* and *Squa*, floral organ identity gene in *Arabidopsis*

thaliana and *Antirrhinum majus* (Cardon 1997). HEN4 is a RNA binding protein in *Arabidopsis thaliana* that involved into RNA processing of floral organ identity gene *AG* (Cheng 2003).

Discussion

The quality of the subtracted cDNA library

When total RNA acts as the starting material, a key factor affecting the quality of the subtracted cDNA library is the circle sum adopted in the process of cDNA amplification, which should meet the need of cDNA amount in subsequent procedure and avoid background as possible especially that from genes with high abundance. According to Clotech protocol, 17–19 is a reasonable sum range with starting material of 0.25–1 μg total RNA, and 18 is the optimized result in this report.

Among the 150 clones sequenced, besides 25 fragments homologous with sequences of unknown function in the public database, 8 shows no significant homology and 35 shows low homology, which seem to be due to short length, or due to large part of UTR. And another possibility is due to the genome identity (some genes only exist in tree genome). Some ESTs in this cDNA library belong to regulators usually with low abundance in cDNA, which implicate a successfully uniformed process.

In this study, gel excising was adopted to modify the result of blastX which might lost some information in the library, but it could be efficiency to screen valuable ESTs.

Relationship between gene expression and development

To enrich genes with differential expression in each development stage, this subtracted cDNA library is constructed according to temporal interval. To some extent, the sequencing result showed the background of gene expression throughout the period of sampling. During the 34 d of sampling, the female inflorescence went through several development stages such as formation of floral meristem, specification of floral organ, impregnation, seed maturation and final senescence. Expression of some genes (*SBP, HEN4, NPG1* etc.) confirmed these events.

Both AP1 and AG are MADS box protein that play a dual role in floral development in *Arabidopsis thaliana*, i.e. AP1 activates floral homeotic gene expression and specifies sepal and petal identity (Ng 2001), while AG controls floral meristem determinacy (Mizukami 1997) and specifies stamen and carpel identity. Expression of homology of *SBP* and *HEN4* should be earlier than *BpMADS3* and *BpMADS6* (homolgy of *AP1* and *AG* in *Betula pendula*). It is indicated that their expression would be durable like BpSPL1 did in *Betula pendula* reported before and AG did in *Arabidopsis thaliana*. It is required for analysis of function about the two regulators.

References

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. 1995. Short protocols in molecular biology [M] (Chinese version in 1998). Beijing: Science Press, p126–127.

Cardon, G.H., Hohmann, S., Nettesheim, K., Saedler, H., Huijser, P. 1997. Functional analysis of the Arabidopsis thaliana SBP-box gene SPL3: a novel gene involved in the floral transition [J]. Plant J., **12**(2): 367–377.

Cheng, Y., Kato, N., Wang, W., Li, J., Chen, X. 2003. Two RNA binding proteins, HEN4 and HUA1, act in the processing of AGAMOUS pre-mRNA in *Arabidopsis thaliana* [J]. Dev. Cell, **4**(1): 53–66.

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C., van Tunen, A.J. 1995. The petunia MADS box gene FBP11 determines ovule identity [J]. Plant Cell, **7**(11): 1859–1868.

Cone, E.S., Meyerowitz, E.M. 1991. The war of the whorls: Genetics interactions controlling flower development [J]. Nature, **353**: 31–37.

Elo, A., Lemmetyinen, J., Turunen, M.L., Tikka, L., Sopanen, T. 2001. Three MADS-box genes similar to APETALA1 and FRUITFULL from silver birch (*Betula pendula*) [J]. Physiol Plant, **112**(1): 95–103.

Felton, G.W., Donato, K.K., Del Vecchio, R.J., Duffey, S.S. 1989. Activation of foliar oxidases by insect feeding reduces nutritive quality of dietary protein of foliage for noctuid herbivores [J]. J Chem Ecol, **15**: 2667–2693

Galan, J.M., Peter, M. 1999. Ubiquitin-dependent degradation of multiple F-box proteins by an autocatalytic mechanism [J]. Proc. Natl. Acad. Sci. USA, **96**(16): 9124–9133.

Golovkin, M., Reddy, A.S. 2003. A calmodulin-binding protein from Arabidopsis has an essential role in pollen germination [J]. Proc. Natl. Acad. Sci. USA, **100**(18): 10558–10563.

Jarvinen, P., Lemmetyinen, J., Savolainen, O., Sopanen, T. 2003. DNA sequence variation in BpMADS2 gene in two populations of *Betula pendula* [J]. Mol. Ecol., **12**(2): 369–384.

Koning, A.J., Tanimoto, E.Y., Kiehne, K., Rost, T., Comai, L. 1991. Cell-specific expression of plant histone H2A genes [J]. Plant Cell, **3**(7): 657–665.

Lannenpaa, M., Janonen, I., Holtta-Vuori, M., Gardemeister, M., Porali, I., Sopanen, T. 2004. A new SBP-box gene BpSPL1 in silver birch (*Betula pendula*) [J]. Physiol. Plant, **120**(3): 491–500.

Lauber, M.H., Waizenegger, I., Steinmann, T., Schwarz, H., Mayer, U., Hwang, I., Lukowitz, W., Jurgens, G. 1997. The Arabidopsis KNOLLE protein is a cytokinesis-specific syntaxin [J]. J Cell Biol., **139**(6): 1485–1493.

Lemmetyinen, J., Hassinen, M., Elo, A., Porali, I., Keinonen, K., Makela, H., Sopanen, T. 2004. Functional characterization of SEPALLATA3 and AGAMOUS orthologues in silver birch [J]. Physiol Plant, **121**(1): 149–162.

Liu, G., Sanchez-Fernandez, R., Li, Z.S., Rea, P.A. 2001. Enhanced multispecificity of arabidopsis vacuolar multidrug resistance-associated protein-type ATP-binding cassette transporter, AtMRP2 [J]. J Biol Chem., **276**(12): 8648–8656.

Lu, Y.P., Li, Z.S., Drozdowicz, Y.M., Hortensteiner, S., Martinoia, E., Rea, P.A. 1998. AtMRP2, an Arabidopsis ATP binding cassette transporter able to transport glutathione S-conjugates and chlorophyll catabolites: functional comparisons with Atmrp1 [J]. Plant Cell, **10**(2): 267–282.

McKinney, E.C., Kandasamy, M.K., Meagher, R.B. 2002. Arabidopsis contains ancient classes of differentially expressed actin-related protein genes [J]. Plant Physiol., **128**(3): 997–1007.

Mizukami, Y., Ma, H. 1997. Determination of Arabidopsis floral meristem identity by AGAMOUS [J]. Plant Cell, **9**(3): 393–408.

Ng, M., Yanofsky, M.F. 2001. Activation of the *Arabidopsis* B class homeotic genes by APETALA1 [J]. Plant Cell, **13**(4):739–753.

Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E., Yanofsky, M.F. 2000. B and C floral organ identity functions require SEPALLATA MADS-box genes [J]. Nature, **405**(6783): 200–203.

Pelttari, J., Hoja, M.R., Yuan, L., Liu, J.G., Brundell, E., Moens, P., Santucci-Darmanin, S., Jessberger, R., Barbero, J.L., Heyting, C., Hoog, C. 2001. A meiotic chromosomal core consisting of cohesin complex proteins recruits DNA recombination proteins and promotes synapsis in the absence of an axial element in mammalian meiotic cells [J]. Mol. Cell Biol., **21**(16): 5667–5677.

Sirko, A., Brodzik, R. 2000. Plant ureases: roles and regulation [J]. Acta Biochim Pol., **47**(4):1189–1195.

Su Xiumin, Wu Fengmao, Hong Tao, Wu Yueliang, Shi Chengjun, Wang Liming. 2000. Technology of anthesis inducing of *Betula platyphylla* [J]. Journal of Liaoning Forestry Science & Technology, **3**: 47. (in Chinese)

Xu, H., Swoboda, I., Bhalla, P.L., Singh, M.B. 1999. Male gametic cell-specific expression of H2A and H3 histone genes [J]. Plant Mol. Biol., **39**(3): 607–614.

Yanagisawa, S. 2002. The Dof family of plant transcription factors [J]. Trends Plant Sci., **7**(12): 555–560.